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ANTITUMORAL AND ANTIVIRAL PEPTIDES

Field of the Invention

The proposed invention relates to proteins and peptides exhibiting antitumoral and antiviral properties, as well as to drugs based thereon.

Background of the Invention

Known are antitumoral peptides of bleomycine group (1). Bleomycines provide a direct cytotoxic action on tumor cells, however, the possibility of their clinic use is restricted by pronounced side effects, first of all onto lungs and kidneys.

Known is use of recombinant proteins of interferon group as activators of antitumoral immunity and inhibitors of tumor cell proliferation. Interferons are used for treating the multiple myeloma (2), Hodgkin's disease (3), myeloid leukemia (4). However, high cost of interferons makes them inaccessible for wide clinical use. Another limitations are side effects associated with possible pyrogenecity, immunogenicity and other undesirable properties of recombinant interferon.

Known are suggestions to use peptide inducers of apoptosis as potential antitumoral drugs (5). However, clinical trends of this direction are still unexplored. Currently, several cytokine-type protein compounds are on the stage of development and clinical testing as antitumoral remedies (6). The most prominent is the use of interleukin-2, but high cost and toxicity of the recombinant interleukin-2 limit its wide use in oncology practice.

Known are suggestions to use hemocyanin and arylphorin proteins as activators of immune response and antitumoral agents (7).

In spite of presence of aforementioned and other elaborations described in the literature, a therapy of oncological diseases remains still ineffective and is prac-

tically always highly toxic and expensive. Therefore, searches of new approaches to tumor therapy remain still one of the most important problems of modern medicine.

Alloferons are known as immunomodulating peptides (8). Therapy of viral infections is the main field of use of said alloferons. At the same time, antitumoral properties of said alloferons based upon activation of antitumoral immunity mechanisms, namely interferons and natural killer cells, are known (9). Alloferons are the closest analogues of the present invention in their chemical structure and action mechanism.

Summary of the Invention

Experimental research of the antitumoral activity of the alloferon has revealed that said peptide depresses the growth of mice tumor syngenic graft, and thereby it can be attributed to promising antitumoral agents. Effect of alloferon is realized at the level of systemic response of organism onto the grafted tumor. At the same time, an effect of alloferon onto tumor proliferation at the cell level seems to be more complicated. Particularly, *in vitro* experiments have shown that the alloferon, depending on its concentration in cultural media, may both inhibit (at high concentrations) and stimulate (at low concentrations) proliferation of tumor cells. Presence of growth-stimulating activity restricts the possibility of use the alloferon for tumor therapy, where depressing of malignizated cell proliferation is the main object of the treatment.

The object of the present invention is to provide drugs exhibiting a reduced growth-stimulating activity and enhanced anti-proliferational and cytotoxic activities towards tumor cells, while keeping the immunomodulating mechanism of alloferon effect.

For this purpose, a new family of peptides was developed, which peptides differ from alloferon and other bioactive compounds in their structure, mechanism of action, and therapeutic effect being achieved.

The claimed group of compounds relates to linear peptides which structure is represented by the following formula:

X_1 Trp Gly Gln X_2 (1)

wherein X_1 is absent, or comprises no less than 1 amino acid,

X_2 is absent, or comprises no less than 1 amino acid.

When developing the present invention, peptide allostatin-1 (SEQ ID NO 1) presented in Table 1 was used as a basic structure. Allostatin-1 has been synthesized by solid-phase synthesis technique and used for studies of biological and therapeutic activity of proposed peptides. Results of the studies are summarized in following examples. The studies have revealed that said peptide has antitumoral activity based on the direct suppression of proliferation of tumor cells and reinforcement of certain links of antitumoral immunity.

During computer analysis of databases containing peptide structures and properties it has been determined that said compound relates to a new family of bioactive peptides previously unknown. Original structure of the proposed peptides ensures the achievement of a new technical level, the possibility of effective suppression of tumor growth and treatment of oncological diseases thereupon.

Analysis of homology of allostatin-1 amino acid sequences and known peptides and proteins, carried out using BLAST SEARCH program upon the data of SWISSPROT database, has revealed several structural analogues of the proposed peptides. These data are summarized in the Table 1.

Revealed sequences with high homology level relative to the allostatin-1, from the structure, functions and origin point of view, belong to prion proteins

(PrP), i.e., compounds of a similar group. Prion proteins (prions) are produced by cells of different tissues of many kinds of animals, including human and other mammals. Normal functions of prions are still not sufficiently explored. At the same time it is known that under certain conditions prions can undergo the conformational changes, resulting in pathological scrapie-isoform which is responsible for propagation of some neurodegenerating diseases. Usually, mature prion protein comprises more than 200 amino acid residues. Pathological properties of prions are connected with fragments homologous to 114-134 PrP I fragment of a bull, particularly to amyloid hydrophobic region AGAAAAGA of said fragment (10). Allostatin-1 is homologous to repeated sites 64-75, 72-83, 80-91, 87-98, 96-108 but structurally it is completely distinguishing from the site 114-134 PrP I. Close structural similarity between said sites and proposed peptides (e.g., 11 of 13 aminoacids (84 %) of 96-108 PrP I site of a bull are similar to allostatin) assumes also the similarity of biological activity. Thus, very probably, one can suppose that fragments of mammal prions homologous to proposed antitumoral peptides, exhibit also similar type of antitumoral activity. Mechanism of probable antitumoral action of said fragments is unknown but some facts suggest that said prions pertain to regulation of T-lymphocytes' activity (11). In turn, T-lymphocytes play the main part in reactions of antitumoral immunity.

Structural and functional similarity to fragments of mammal prions allows to pick out potentially variable sites of the sequence of proposed peptides, in which sites the substitution in composition and ordering of amino acids will not effect essentially onto functional properties of the molecule as a whole. Taking into account the distribution of variable and conservative sites of amino acid sequences shown in the Table 1, the general structural formula (1) includes two variable zones X_1 and X_2 divided by conservative sequence of tryptophan, glycine and glutamine

amino acids (Trp-Gly-Gln). Variable site X_1 is absent or it can comprise up to 5 aminoacids or more. Variable region X_2 is absent, or it can comprise up to 7 amino acids or more. Here, the proposed peptides may be included as a functional part of other proteins and polypeptides into larger amino acid sequences, e.g., prion proteins having chain length of 250-300 amino acids.

Compounds of the proposed structure, which are represented by allostatin-1, are synthesized by solid-phase synthesis technique and characterized by HPLC and mass-spectrometry. Said compounds can be obtained in a form of ethers, salts, amides or other pharmaceutically acceptable derivatives. Besides the chemical way of synthesis, proposed peptides can be obtained by genetic engineering techniques or recovered from natural sources.

Other structural analogues of the proposed peptides are alloferons, which have general structural formula described in the patent (12). Results of comparative analysis of structural formulae of alloferons and proposed peptides, allostatins, are presented in the Table 2 and Table 3. Table 2 compares structures of two typical representatives of peptide family, alloferon-1 (SEQ ID NO 12) and allostatin-1 (SEQ ID NO 1). Based on the comparison one can see that said peptides differ from each other in amino acids in positions 6 and 11, namely histidine and valine of alloferon-1, and tryptophan and threonine of allostatin-1, respectively. According to RU 2172322, positions 6 and 11 are invariable part and typical feature of all alloferons. Replacement of amino acids in these positions with tryptophan and threonine results in desired modification of biological activity and therapeutic effect, as following examples confirm.

Comparison of general structural formulae (Table 3) shows that the compositions of conservative sites and arrangement of variable sites of allostatin and alloferon molecules have a qualitative difference. For this reason, they can be rated

as two different peptide families.

Examples confirming the possibility for realization of the invention

EXAMPLE 1. Allostatin-1 synthesis

Peptide consisting of 13 aminoacids corresponding to the allostatin-1 structure, was synthesized by solid-phase synthesis technique using the automatic multichannel synthesizer Multisyntech GmbH Witten and Fmoc-(N-[9-fluorenyl]methoxycarbonyl)-substituted amino acids. Purification of the synthesized peptide was carried out by technique of reversed-phase HPLC using Shimazu LC8 chromatograph equipped with 10 mm Chromasil C18 column. Purity of thus obtained peptide was also controlled by HPLC method (Fig. 1). Correctness of the synthesis is confirmed by MALDI-TOF mass-spectroscopy method using Finnigan TSQ 7000 device (Fig. 2). Mass of the peptide established experimentally corresponds to the calculated one, and deviations are within the range of measurement error.

EXAMPLE 2. *In vitro* influence of allostatin on tumor cells proliferation

Object of instant experiments is the comparative analysis of influence of allostatin and alloferon on tumor cells proliferation. Effects of allostatin-1 and alloferon-1 in concentrations 0.001, 0.01, 0.1, 1, and 10 microgram per ml on proliferative activity in a mass culture of R388D1-type tumor cells were compared. 5000 cells suspended in 2 ml of RPMI 164 medium were seed into wells of 24-socket plates. Medium used for the experiments comprises 5 % fetal calf serum produced by firm "Biolot". Preparations were introduced into wells in 0.2 ml of the same medium immediately after inoculation, and, in control, equivalent amount of the

medium was introduced without medications. Number of cells in 1 ml of incubating medium was estimated by means of hemocytometric camera. Average number of cells in 1 ml of incubating medium after 21, 44, 99 and 144 hours after beginning of the experiment was estimated on the base of 3 independent measurements.

Figure 3 presents a typical view of allostatin and alloferon influence on growth dynamics of tumor cell population. As an evaluation indicator of anti-proliferative activity of the medication, the value of a growth multiplicity of the population for 90 hours was selected, which value is determined as a ratio of a number of cells in a well in the beginning and at the end of the incubation time. During said period, control number of cells increased approximately 30 times. When medications were used, number of cells and rate of proliferation, respectively, were decreased in a dose-dependent manner. Here, anti-proliferative activity of allostatin 3-7 times exceeds the one of alloferon when concentrations of 0.001-1 mkg/ml were used. Allostatin in a concentration of 10 microgram/ml has almost completely stopped the growth of the tumor cell population over the observation period.

Thus, this example 2 demonstrates the presence of anti-proliferative activity of allostatin, and its advantage in comparison with alloferon.

EXAMPLE 3. *In vitro* interaction of allostatin and antitumoral cytostatics

Example 3 shows materials that demonstrate an interaction of allostatin and typical cytostatic, cyclophosphamide, regarding suppression of clonogenic activity of tumor cells. Clonogenic activity index allows to determine the part of tumor cells from general pool which are able to produce viable clones and thus take part in growth and proliferation of the tumor. The main purpose of chemotherapy is a destruction of such actively proliferating cells.

Experimental technique can be summarized as following. Cells of lymphoid

neoplasm of a mouse (line R388D1) were cultivated in RPMI 1640 medium containing glutamine, gentamicin and 10% of embryonic calf serum "High clone". 100 R388D1cells in 1 ml of said medium were seed into each well of 24-socket culture plates. After that, 0.1 ml of the medium with or without testing preparation (control wells) was introduced into each well. Each embodiment of the experiment was independently repeated for 3 times. Numbers of clones were counted after 7 days from the cultivation beginning.

As one can see (Table 4), about 15 % of tumor cells resulted in viable clones under conditions of this experiment. Neither cyclophosphamide, nor allostatin taken separately, had a significant influence upon the cloning process. At the same time, their combination has significantly decreased a clonogenic activity of tumor cells, proportionally to allostatin dose.

The present example shows that allostatin has perspectives for use in combined tumor chemotherapy in combination with cytostatics of cyclophosphamide type.

EXAMPLE 4. Antitumoral action of allostatin onto models of transplanted tumors in mice

Each of laboratory mouse (line DBA-1) was subcutaneously injected by 3000 tumor cells of the syngeneic line R388D1. Next day mice were separated into 4 experimental groups. Mice of the first group have got only allostatin in a subcutaneous way in a dosage on 25 microgram at 4, 11 and 18 day after tumor cell transplantation. Second group has got a combination of cytostatic agents, cyclophosphamide (0.56 mg), doxorubicine (0.036 mg) and vincrystine (1.05 microgram) on the day of transplantation and after 7, 14 and 21 days. Third group has got allostatin and combination of cytostatic agents in the same way. Mice of the fourth group

(control group) were injected by the solvent (0.9 % NaCl) in the same days.

In the control group palpable tumors in grafted areas became apparent after 20 days, after 25 days all mice have had typical subcutaneous tumors of 5-26 mm in a diameter (Fig. 4). In groups where mice have got allostatin or cytostatics separately, tumors were formed after some delays, and moderate part of animals has not exhibited tumor formation during observation time. At the same time, strong and in the most cases irreversible antitumoral action was detected when combination of allostatin and cytostatics was used. Only 40 % of mice of this group have exhibited formed tumors during observation period ($P<0.001$ with respect to the control group, and $P<0.05$ with respect to the group administered cytostatics only).

This example, as the Example 3 above, indicates that the allostatin have a pronounced antitumoral effect when it is used in the combination with typical drugs for chemotherapy, widely applied for treating leucosis and other oncological diseases.

Example 5. Immunomodulating (interferonogenic) activity of allostatin

Action mechanism of immunomodulators, which alloferon belongs to, is associated with induction of interferon synthesis provided by blood leucocytes (Chernysh et al., Proceedings of National Academy of Science, 2002, 99, p. 12628-12632). One of the purposes of present invention was to keep immunomodulating action of allostatins in the range of biological activity thereof. The present example illustrates an immunomodulating activity of allostatin-1 using a model of interferon *in vitro* synthesis provided by leucocytes of a human.

Samples of donated blood were mixed with an aqueous solution of testing sample and culture medium in the ratio of 1:1:8. Final concentration of samples in incubation mixture was 0 (control), 0.01, 0.1, 1, or 10 microgram/ml, for different

embodiments of the example. This mixture was incubated at 37 °C for 24 hours in a CO_2-thermostat. Then, blood cells were precipitated by centrifugation. After that, serial dilutions of the obtained supernatant were placed into wells of 96-socket plates, covered by monolayer of a test-culture of L-41 cells, and then incubated for 24 hours under the same conditions. Then, the cell monolayer was infected by vesicular stomatitis virus in a dose equal to 100 LD_{50} (dose caused in 50 % death of monolayer cells) and incubated at 37 °C for 18 hours. Then, cells were visualized by 0.1% solution of a crystal-purple dye. Part of monolayer destroyed by the virus was determined using measurements of optical density of extracted dye at the wavelength of 590 nm. Values thus obtained were compared with an effect of reference sample of alpha-interferon, and obtained titer of interferon was calculated in IU-units of antiviral activity of alpha-interferon. Results of studies of 6 donated blood samples taken at two analytical repetitions (12 measurements for each point), are summarized in Fig. 5.

The obtained results indicate that interferonogenic activities of allostatin and alloferon have no significant difference. Hence, allostatin acquires specific properties which are suitable to use it as an antitumoral agent, and at the same time, allostatin keeps the immunomodulating activity intrinsic to alloferon. Thus, allostatin can be used in oncology (and in other fields of use) as a dual-purpose drug, both direct (cytotoxic and antiproliferative effects, potentiating of cytostatics' effect) and indirect (immunomodulating) actions.

EXAMPLE 6. Antiviral activity of allostatin

Lethal influenza virus infection of wild-type white mice (body mass of 14-16 g) of both sexes was used as a model in the studies of antiviral action of allostatin. Influenza virus A/Aichi/2/68 (H3N2) adapted to white mice was used in the present study. Allostatin and alloferon were dissolved in distilled water and then 0.25 ml

was subcutaneously injected to each animal on the basis of 25 microgram per mouse (1.5 mg/kg of a body). Distilled water was used as a placebo in the control group. Preventive scheme of injection was used to determine the antiviral activity of drugs: single injection of the drug was done 24 hours before infection. Virus doses of 3 and 30 LD_{50} were introduced intranasally into animals under light ether anesthesia. Each testing group comprised of 10 mice. Observation of animals lasted during 14 days. Death-rate of the animals was registered both for experimental and control groups. Results of the experiment are presented in a Table 5. Both of drugs provided similar effective protection against lethal influenza virus infection of mice.

Thus, allostatin keeps an antiviral activity typical for alloferon. One can suppose thereupon that allostatin can be used as antiviral drug, as well as alloferon. The most advisable use thereof is the use instead of alloferon in border-line cases of viral and oncological pathology, e.g. in the case of tumors of viral ethiology or for the purpose of treating viral infections of cancer patients.

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12. RU 2172322

Best variant of the invention embodiment

Antitumoral and antiviral peptide comprising 13 aminoacids and corresponding to the structure of allostatin 1 is represented in the Example 1 as the best variant since it most completely discloses therapeutic efficacy of the declared peptide class according to the laboratory assessment. The peptide was synthesized by solid-phase synthesis technique using the automatic multi-channel synthesizer Multisyntech GmbH Witten and Fmoc-(N-[9-fluorenyl]methoxycarbonyl)-substituted amino acids. Purification of the synthesized peptide was carried out by technique of reversed-phase HPLC using Shimazu LC8 chromatograph equipped with 10 mm Chromasil C18 column. Purity of thus obtained peptide was also controlled by HPLC method (Fig. 1). Correctness of the synthesis is confirmed by MALDI-TOF mass-spectroscopy method using Finnigan TSQ 7000 device (Fig. 2). Mass of the peptide established experimentally corresponds to the calculated one, and deviations are within the range of measurement error.

Industrial applicability

Industrial applicability of the invention is confirmed by the results of laboratory studies and calculations that represented in the examples 1-6 and the Tables 4 and 5 mentioned below. These materials show that allostatin administration allows to suppress tumor cells proliferation and to eliminate them by the system of the organism immunological surveillance, which is a main goal of oncological diseases therapy and prophylaxis. Similarly, represented materials confirm the invention applicability to the therapy of viral infections by means of stimulation of antiviral immunity mechanisms. A method of declared peptides synthesis described in the application materials is available to enlargement in industrial conditions.